PA. ENT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

To:

NOAM, Meir
P.O. Box 34335
91342 Jerusalem
ISRAËL

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year)
27 October 1998 (27.10.98)

Applicant's or agent's file reference
a511-34-Y

IMPORTANT NOTIFICATION

International application No.
PCT/IL97/00180

International filing date (day/month/year)
04 June 1997 (04.06.97)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
NOAM, Meir
P.O. Box 32081
91320 Jerusalem
Israel

State of Nationality

State of Residence

Telephone No.
972-2-6232768

Facsimile No.
972-2-6250907

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
NOAM, Meir
P.O. Box 34335
91342 Jerusalem
Israel

State of Nationality

State of Residence

Telephone No.
972-2-6518880

Facsimile No.
972-2-6523336

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Jocelyne Rey-Millet

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

002303423

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as designated Office

Date of mailing:
12 March 1998 (12.03.98)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/IL97/00180	WO97/46259

CORRECTED VERSION
VERSION CORRIGEE

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

1935972

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
A511-34-Y		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IL97/00180	04/06/1997	04/06/1996
International Patent Classification (IPC) or national classification and IPC		
A61K47/48		
Applicant		
YISSUM RESEARCH DEVELOPMENT COMPANY ... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/01/1998	07.07.98
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0. Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Ludwig, G Telephone No. (+49-89) 2399-8698

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IL97/00180

I. Basis of the r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-18	as originally filed				

Claims, No.:

6-20	as originally filed				
1-5	as received on	24/06/1998	with letter of	16/06/1998	

Drawings, sheets:

1/8-8/8	as originally filed				

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 3-20.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 3-6 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-2
	No:	Claims
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-2
Industrial applicability (IA)	Yes:	Claims 1-2
	No:	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

s separate sh t

The following documents (D) are referred to in this report:

D1: WO 93/15751
D2: WO 90/0799

1. Documents D1 and D2 disclose chemical conjugates of gonadotropin releasing hormone (GnRH) and the toxin Pseudomonas exotoxin (PE) for the treatment of breast cancer, prostate cancer and endometriosis (D1) and of sex related cancers of the breast and prostate (D2), respectively

   (D1: abstract; page 10, paragraph 1; page 25, line 31; age 26, lines 28-29, pages 25-26; claims 1 and 8-11, page 5, line 16 - page 6, paragraph 1;
   D2: abstract, page 1, paragraph 1 and claims).

2. In the introduction of claims 3-4 claim a targeted fused chimeric toxin according to claim 1, i.e. a **protein** is claimed. In contrast, the rest of these claims refers to the cell targeting moiety which is part of the toxin as being an **oligonucleotide**. It is not known what is intended by these claims.

   This objection applies applies, mutatis mutandis, to claims 5-6 which are confusing and cannnot be understood.

3. Genetically engineered fusion proteins are standard in the art of biochemistry of targeted conjugates (D1, page 5, paragraph 2; page 6, paragraphs 1-2; page 7, paragraph 1; page 8, lines 4-10).

   Having regard to documents D1 and D2 as characterized above nothing inventive can therefore be seen in claim 1 in view of the problem to be solved by the application, i.e. to provide alternative GnRH-PE conjugates for tumour treatment.

4. Due to its reference to endometrial island cells (malignant adenocarcinoma cells) claim 2 is not regarded as inventive vis-a-vis document D1 (documents D1 and D2).

5. For the assessment of the presently worded claims 9-20 on the question whether it is industrially applicable, no unified criteria exist in the PCT. In the Contracting States of the PCT the patentability of such a claim can also depend on its formulation.
Accordingly, the applicant is informed that under the EPC these claims would not be allowable (Art. 52(4) & 52(1) EPC).

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 19 January 1998 (19.01.98)	
International application No. PCT/IL97/00180	**Applicant's or agent's file reference** a511-34-Y
International filing date (day/month/year) 04 June 1997 (04.06.97)	**Priority date** (day/month/year) 04 June 1996 (04.06.96)
Applicant YARKONI, Shai et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   04 January 1998 (04.01.98)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Carlos Roy
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

WO 97/46259
PCT/IL97/00180

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

NOAM, Meir
P.O. Box 32081
91320 Jerusalem
ISRAËL

Date of mailing (day/month/year) 11 December 1997 (11.12.97)	
Applicant's or agent's file reference a511-34-Y	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/IL97/00180	04 June 1997 (04.06.97)	04 June 1996 (04.06.96)

Applicant YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,BR,CA,CN,EP,IL,JP,KP,KR,NO,PL,SK,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AL,AM,AP,AT,AZ,BB,BG,BY,CH,CZ,DE,DK,EA,EE,ES,FI,GB,GE,HU,IS,KE,KG,KZ,LK,LR,LS,LT, LU,LV,MD,MG,MK,MN,MW,MX,NZ,OA,PT,RO,RU,SD,SE,SG,SI,TJ,TM,TR,TT,UA,UG,UZ,VN

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   11 December 1997 (11.12.97) under No. WO 97/46259

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rul s 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
a511-34-Y		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IL 97/00180	04/06/1997	04/06/1996
Applicant		
YISSUM RESEARCH DEVELOPMENT COMPANY ... et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established by this Authority to read as follows:

   CHIMERIC TOXIN FOR TARGETED THERAPY

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 97/00180

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 9-20
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTER IONAL SEARCH REPORT

Int[redacted]nal Application No
PCT/IL 97/00180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NECHUSHTAN, AMOTZ ET AL: "Adenocarcinoma cells are targeted by the new GnRH -PE66 chimeric toxin through specific gonadotropin-releasing hormone binding sites" J. BIOL. CHEM. (1997), 272(17), 11597-11603 CODEN: JBCHA3;ISSN: 0021-9258, 1997, XP002051492 see abstract; figures ---	1-20
X	WO 93 15751 A (MERCK & CO INC) 19 August 1993 cited in the application see claims ---	1-20
P,A	WO 96 24675 A (UNIV SASKATCHEWAN) 15 August 1996 see claims 1-5 ---	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 January 1998	4. 02. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 22364 A (YISSUM RES DEV CO ;FISHMAN ALA (IL); YARKONI SHAI (IL); LORBERBOUM) 26 June 1997 see claims 1,2,7 ---	1-20
X	WO 90 09799 A (UNIV COLORADO RES) 7 September 1990 see page 1, line 3 - line 9 see page 9, line 2 - line 27 see page 14, line 15 - line 34 ---	1-20
X	RUSIECKI, V. K. ET AL: "GnRH -toxin chimera as chemosterilants: Synthesis and conjugation of GnRH analogs to truncated bacterial toxins" PEPT. 1994, PROC. EUR. PEPT. SYMP., 23RD (1995), MEETING DATE 1994, 765-766. EDITOR(S): MAIA, HERNANI L. S. PUBLISHER: ESCOM, LEIDEN, NETH. CODEN: 63MBAO, 1995, XP002051493 see the whole document -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IL 97/00180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9315751 A	19-08-93	AU 3665293 A	03-09-93
		ZA 9300988 A	20-09-93
WO 9624675 A	15-08-96	AU 4477796 A	27-08-96
		CA 2212054 A	15-08-96
		EP 0808369 A	26-11-97
WO 9722364 A	26-06-97	AU 1070797 A	14-07-97
WO 9009799 A	07-09-90	AU 5186090 A	26-09-90
		US 5631229 A	20-05-97
		US 5492893 A	20-02-96
		US 5488036 A	30-01-96
		US 5378688 A	03-01-95

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48

A2

(11) International Publication Number: **WO 97/46259**

(43) International Publication Date: 11 December 1997 (11.12.97)

(21) International Application Number: PCT/IL97/00180

(22) International Filing Date: 4 June 1997 (04.06.97)

(30) Priority Data:
118570 4 June 1996 (04.06.96) IL

(71) Applicant *(for all designated States except US)*: YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM [IL/IL]; Jabotinsky Street 46, 91042 Jerusalem (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: YARKONI, Shai [IL/IL]; Halamed Heh Street 33, 44395 Kfar Saba (IL). NECHUSHTAN, Amotz [IL/IL]; Habanim Street 31, 47223 Ramat Hasharon (IL). LORBERBOUM-GALSKI, Haya [IL/IL]; Bar Kochva Street 72/3, 97875 Jerusalem (IL). MARIANOVSKI, Irina [IL/IL]; Medical School of Hadasa, Dept. of Cellular Biochemistry, Ein Karem, 91120 Jerusalem (IL).

(74) Agent: NOAM, Meir; P.O. Box 32081, 91320 Jerusalem (IL).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: CHIMERIC TOXINS FOR TARGETED THERAPY

(57) Abstract

The present invention relates particularly to neoplastic cells targeted chimeric toxins comprising of cell targeting moieties and cell killing moieties for recognizing and for destroying the neoplastic cells, wherein the cell targeting moieties consist of gonadotropin releasing hormone homologues and the cell killing moieties consist of Pseudomonas Exotoxin A. The present invention further relates to pharmaceutical compositions containing as an active ingredient these neoplastic cells targeted chimeric toxins and to a method for the production of these chimeric toxins. The said invention also relates to a method for cancer therapy, treating malignant carcinoma cells and benign hyperplasia including uterine lyomyoma cells, extra uterian endometrial island cells, benign hyperplasia of prostate and breast and pituitary tumor adenoma cells, by the use of the above-mentioned chimeric toxins.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
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CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
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CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CHIMERIC TOXINS FOR TARGETED THERAPY

Field of the invention

The present invention relates generally to therapeutic agents useful particularly in cancer targeted therapy but also in treating malignant carcinomas such as breast, colon, hepatic, ovarian and renal carcinomas and treating benign tumors of the uterus, hyperplasia, endometriosis, BPH, polycystic disease of the breast and pituitary adenomas.

More specifically the said invention relates to Pseudomonas Exotoxin based chimeric toxins aimed at those neoplastic cells bearing gonadotropin releasing hormone binding sites. The present invention further relates to pharmaceutical compositions comprising as an active ingredient the above mentioned neoplastic cell targeted chimeric toxins. Furthermore the present invention relates to a method for the production of said chimeric toxins. These chimeric proteins, according to the present invention, are comprised of cell targeting moieties which consist of gonadotropin releasing hormone homologues linked to cell killing moieties which consist, preferably, of the bacterial toxin Pseudomonas Exotoxin A , for recognizing and destroying neoplastic cells bearing gonadotropin releasing hormone binding sites.

Targeting is a term for the selective delivery of chemotherapeutic agents to specific cell populations. It is possible to create chimeric molecules that possess cell targeting and cellular toxin domains. These chimeric molecules function as cell selective poisons by virtue of their abilities to target selective cells and then kill those cells via their toxin component. Pseudomonas Exotoxin A (hereinafter called PE), a bacterial toxin used in construction of such chimeric proteins, acts by irreversibly arresting protein synthesis in eukaryotic cells, resulting in

cell death.

The term "gonadotropin releasing hormone homologues" in this invention relates to the gonadotropin releasing hormone gene itself or its analogues and antagonists.
Also included in the scope of the present invention are salts of the described chimeric proteins. The term "salts" includes both salts of carboxy groups as well as acid addition salts of amino groups of the protein molecule. The invention further relates to pharmaceutical compositions comprising the chimeric proteins as defined above together with a pharmaceutically acceptable inert carrier. The proteins of the present invention may be administered by methods known in the art for the administeration of proteins.

Background of the invention

Gonadotropin releasing hormone (hereinafter called GnRH) participates in the hypothalamic - pituitary gonadal control of human reproduction. The involvment of GnRH has been demonstrated in several carcinomas and GnRH analogue treatment has been applied in breast, prostatic, pancreatic, endometrial and ovarian cancers (Kadar T. et al. Prostate 12: 229 - 307, 1988). These analogues suppress tumor cell growth in vitro and in vivo. The existence of GnRH binding sites was revealed in the corresponding malignant cells and in well established cell lines (Emons G. et al. J.Clin.Endocrinol.Metab. 77: 1458 - 1464, 1993), though prelimenary results suggest that the GnRH receptor involved may differ from the previously documented receptor (Kadar et al. Biochem. Biophs. Res. Comm. 189: 289 - 295, 1992).

Although GnRH binding sites have been demonstrated in a number of solid tumors and various carcinoma cell lines derived mainly from hormone dependant tissues, their existence in colon or renal carcinoma has not been previously documented. The presence of specific GnRH binding sites in colon, breast, prostate, ovarian endometrium, renal

and liver carcinomas, is shown here. Surprisingly, the specific GnRH binding sites are not limited to hormone-dependant tissues, as indicated by the marked killing of colon carcinoma, renal cell carcinoma and hepatocarcinoma cells.

WO Patent No. WO93/15751 describes various conjugates of GnRH, a linking group and Pseudomonas Toxin A, prepared using the techniques of synthetic organic chemistry, used for the sterilization of animals by killing gonadotrophin releasing cells of the animals pituitary gland.

The present invention describes the construction, by the techniques of genetic enginering, of PE based chimeric toxins, aimed at targeting those neoplastic cells bearing GnRH binding sites. The chimeric toxins of the present invention are fusion proteins and, as such, do not contain a chemical linking group (as in the above mentioned patent). Therefor, they are completely different proteins from the molecules described in the WO patent.

Using different kinds of targeting moieties, a large number of immunotoxins have been generated in the last 20 years by chemical linkage techniques or recombinant DNA technology. The size of these targeting moieties varies widely, ranging from large antibodies to small growth factors, cytokines and antibody fragments.

The ability of large chimeric proteins, as the GnRH-PE constructions described in the present invention, to target cells via a very small portion of the polypeptide (a peptide of ten amino acids, as used as the targeting moiety of the present invention), and yet retain their original functions, namely binding and internalization, open up new possibilities in designing targeted immunotoxins.

Colon, breast, and prostate cancer - three out of the four major malignancies occuring in humans, together with ovarian, endometrium, renal and liver carcinomas, account for more than 50% of cancer related death. The presence of specific GnRH binding sites in all these cancers, may suggest a more general role of GnRH and/or GnRH - like

peptides in the malignant process.

Collectively, these results disclosed what could be considered the Achilles' heel of these malignant growths, a finding which could open up new vistas in the fight against cancer.

In view of their efficient growth inhibition of the above mentioned cancer cells and their specificity regarding the non target cells, the novel GnRH-PE chimeric toxins are promising candidates for cancer treatment.

Summary of the invention

The present invention relates particularly to neoplastic cells targeted chimeric toxins comprising of cell targeting moieties and cell killing moieties for recognizing and for destroying the neoplastic cells, wherein the cell targeting moieties consist of gonadotropin releasing hormone homologues and the cell killing moieties consist of Pseudomonas Exotoxin A. The present invention further relates to pharmaceutical compositions containing as an active ingredient these neoplastic cells targeted chimeric toxins and to a method for the production of these chimeric toxins. The said invention also relates to a method for cancer therapy, treating malignant carcinoma cells and benign hyperplasia including uterine lyomyoma cells, extra uterian endometrial island cells, benign hyperplasia of prostate and breast and pituitary tunor adenoma cells, by the use of the above mentioned chimeric toxins.

Detailed description of the invention

The present invention describes Pseudomonas Exotoxin A (PE) based chimeric toxins constructed by ligating an oligonucleotide encoding ten amino acids of a gonadotropin releasing hormone (GnRH) analog (GnRH coding sequence with tryptophane replacing glycine as the sixth amino acid) upstream to a mutated form of PE (domains I(mutated),II and III) thereby generating GnRH-PE66, and a ten amino acid synthetic GnRH oligomer(GnRH coding sequence with tryptophane replacing glycine as the sixth amino acid) ligated to domains II and III of the PE, thereby generating GnRH-PE40 protein.

The applications, potential markets and commercial advantages of the said chimeric proteins according to the present invention are listed:

These are two main applications:

1) The malignant carcinomas:

Breast, colon, hepatic, ovarian and renal carcinomas were all sensitive to GnRH - PE mediated cytotoxicity. Thus, the potential market for this new chimeric protein includes all carcinoma patients either as a first line of treatment or for patients in which other modalities of treatment had failed.

2) The benign tumors of the uterus and hyperplasia:

This group of pathalogies includes various tissues that are known to be sensitive to GnRH and thus can be targeted by the GnRH-PE chimeric proteins.

a. Uterine - Uterine lyomyoma is the most common benign tumor in women. The uterine myomas are found to carry a large number of GnRH receptors. GnRH analogs are clinically used for down regulation and shrinkage of these myomas. The disadvantage of GnRH analogs is that these compounds can not be used for long periods and the myomas return to their original size after cessation of the treatment. The use of GnRH-PE for the destruction of the myomas can help to avoid what was considered to be iminant hysterectomies, as well as hemostatic drugs taken by these patients. The optional market includes women with fibroid uterus.

b. Endometriosis - Endometrioma: The existence of endometrial tissue out of the uterus leads to the disease called endometriosis which can cause infertility, abdominal pain and even surgical emergencies.

The endometrial islands are known to be very sensitive to hormonal changes. One of the therapeutic modalities found to be clinically effecient is the GnRH analog. Using GnRH-PE the endometrial activity of these islands can be arrested, thereby helping infertile couples as well as women who are undergoing laparotomy for the resection of these endometrial islands. The treatment of both the lyomyoma and the endometria can be administered systematically or locally by either ultra sonic or laparoscopic guided injection into the endometriomal peritoneal cavity or by a slow release into the uterine cavity.

c. Benign Prostatic Hyperplasia (BPH):

The prostatic cells are known to express GnRH receptors and prostatic cancer is successfully treated today with GnRH agonists. The BPH cause severe symptoms of dissuria urinary retention and sometimes can be treated only by prostatectomy. The use of GnRH-PE can therefor replace all prostatectomies procedures carried on prostate hyperplasia that is not malignant.

The potential market is all the elderly men suffering from symptomatic prostate enlargement. GnRH-PE chimeric proteins may be administered systemically or trans uterally.

d. Polycystic disease of the breast:

The mammary cells are also known to express the GnRH receptors. As in the case of BPH, the polycystic disease of the breast may be symptomatic, cause pain and may mimic breast carcinoma. The use of GnRH-PE may eliminate the need for numerous check-ups and needless mamographies and help woman suffering from breast pains and ***** of breast malignancy.

Thhe potential market is a large population of woman in whom polycystic breast disease is diagnosed. GnRH-PE may be administered systemically.

e. Pituitary adenoma: some of the pituitary adenomas are derived from gonadotropic cells. The pituitary adenoma, even though non malignant, can cause a grave prognosis by causing local pressure on vital organs (eyes, brain stem). The trans-sphenoidal surgery used for the pituitary adenoma has many disadvantages including recurrency and neurological sequella. GnRH-PE may be aimed directly against the gonadotropic cells without damaging other functions of the pituitary gland. GnRH-PE chimeric toxin may be administered intra-thecally.

Commercial advantages:

1. The wide variety of tumors that respond to the GnRH-PE chimeric protein.
2. The high selectivity that allows a large therapeutic range.
3. The use of GnRH as a targeting peptide leaving the large population of postmenopausal women in whom the GnRH has no physiological role perfect candidates for the treatment.
4. Its' high specificity enables the systemic administration together with the local effect.
5. The ability to eradicate small populations of cells in a tissue that will not be harmed by itself.

The proteins of the present invention may be administered by methods known in the art for the administeration of proteins. Also included in the scope of the present invention are salts of the described chimeric proteins. The term "salts" includes both salts of carboxy groups as well as acid addition salts of amino groups of the protein molecule. Salts of the carboxy group may be formed by methods known in the art and include both inorganic salts as well as salts with organic bases. The invention further relates to pharmaceutical compositions comprising the chimeric proteins as defined above together with a pharmaceutically acceptable inert carrier. The pharmaceutical composition may be administered by injection (intra-veneous, intra-articular,

sub-cutaneous, intra-muscular, intra-peritoneal) toppical application, oral administration, sustained release, or by any other route including the enteral route.

The said invention will be further described in detail by the following experiments and figures. These experiments and figures do not intend to limit the scope of the invention but to demonstrate and clarify it only.

Description of the Figures:

Figure 1: Construction and expression of the GnRH-PE66 chimeric toxin. A, SDS-PAGE gel and B, immunoblotting analysis of TGnRH-PE66 plasmid expression. Whole cell extract of the lysed bacteria (lane 1). Soluble fraction (lane 2). Insoluble fraction (lane 3). C, construction of TGnRH-PE66 plasmid.

T7 promotor. GnRH analogue peptide. Ampicillin^R

PE664glu. The numbers represent the corresponding amino acids.

Figure 2: The effect of increasing concentrations of GnRH-PE66 on various cell lines.

A: ■ SW-48 colon carcinoma, ● HepG2 hepatocarcinoma, ▲ Caco2 colon carcinoma.

B: ✜ OVCAR3 ovarian carcinoma, ✚ Hela cervix adenocarcinoma, ○ MDA breast carcinoma, ✕ HT-29 colon carcinoma.

Figure 3: The effect of GnRH-PE66 on various primary cultures. A, colon carcinoma primary cultures established from three patients. B, renal cell carcinoma primary culture. C, breast carcinoma primary cultures established from four patients. D, ovarian carcinoma primary cultures established from two patients. E, metastases primary cultures established from the corresponding patients represented in A,C and D by the same symbols. ● colon carcinoma metastases. ✚ breast carcinoma metastases. ✠, ○ two ovarian carcinoma metastases. F, control cells: # leukocytes. ▶bonemarrow. • fibroblasts. ▽colon.

Figure 4: Histopathological diagnosis of primary cultures. A, anti-keratin positive staining of a colon primary culture. B, anti-desmin negative staining of a colon primary culture.

Figure 5: Displacement of [^{125}I] GnRH bound to membranes of SW-48 cells by: ○ GnRH-PE66. ■ GnRH analogue (des-Gly^{10},[d-Ala^{6}]-LHRH).

Figure 6: Purification of GnRH-PE66.

1 - protein marker. 2 - whole cell extract. 3 - soluble fraction. 4 - insoluble fraction after refolding. 5 - after DEAE - Sepharose column. 6 - after Sepharyl S-200 HR column.

Figure 7: Purification of GnRH-PE40:

1 - protein marker. 2 - whole cell extract. 3 - soluble fraction. 4 - insoluble fraction after refolding. 5 - after DEAE - Sepharose column. 6 - after Sepharyl S-200 HR column.

Figure 8: Effects of GnRH-PE chimeric proteins on SW-48 colon carcinoma cell line:

● GnRH-PE66 insoluble fraction after refolding.

▲ GnRH-PE66 purified protein.

● GnRH-PE40 purified protein.

Experiments

1. GnRH-PE66 chimeric toxin construction

A plasmid vector carrying the mutated full length PE gene (pJY3A1136-1,3) (Chandhary V., Jinno Y., Gall M., Fitzgerald D. and Patsan T. J. Biol. Chem. 256, 16306-16310, 1990) was cut with NdeI and Hind III. The insert was a 36 base pair synthetic oligomer consisting of the GnRH coding sequence with tryptophan replacing glycin as the sixth amino acid, was flanked by NdeI (5' end) and HindIII (3' end) restriction sites. The resulting TGnRH-PE66 plasmid was confirmed by restriction endonucleases digestion and DNA sequence analysis (Figure 1c).

2. TGnRH-PE40 plasmid construction

To construct the GnRH-PE40 protein (GnRH-domains II and III of the PE), the TGnRH-PE66 plasmid vector (fig. 1c) was digested with NdeI and BamHI and ligated to a NdeI-BamHI 750bp fragment from the plasmid PHL-906 (Fishman A., Bar-Kana Y., Steinberger I. and Lorberboum-Galski H. Biochemistry 33, 6235-6243, 1994) along with an insert which is a 36 base pair synthetic oligomer consisting of the GnRH coding sequence with tryptophan replacing glycin as the sixth amino acid, flanked by NdeI (5' end) and HindIII (3' end) restriction sites. The resulting TGnRH-PE40 plasmid was confirmed by restriction endonucleases digestion and DNA sequence analysis.

3. Protein expression

Protein expression method was the same for GnRH-PE40 and GnRH-PE66, unless mentioned. Escherichia coli strain BL21 (DE3) carrying the plasmid TGnRH-PE66 was grown in LB medium contai`ning ampicillin (100µg/ml) and Escherichia coli strain BL21 (DE3) carrying the plasmid GnRH-PE40 was grown in super LB medium containing ampicilin (50 µg/ml). After reaching an A600 value of 1.5 - 1.7, the cultures were induced 90 minutes for GnRH-PE66 and over night for GnRH-PE40, at 37°C with 1 mM isopropyl-d-thiogalactoside (IPTG). Cells were collected by centrifugation and the pellet was incubated at -70°C for several hours.
The frozen pellet was thawed and suspended in lysis buffer (50mM Tris HCl, pH 8.0, 1mM EDTA and lysozyme 0.2 mg/ml), followed by sonication (3 x 30 seconds) and centrifugation at 35,000 x g for 30 minutes. The supernatant (soluble fraction) was removed and the pellet (insoluble fraction) served as the source for the chimeric proteins and for their purification.
Analysis of the insoluble fraction by SDS/PAGE gel electrophoresis revealed a major band (70%) with an expected molecular mass of 67kDa, corresponding to the chimeric protein, and two major unrelated bacterial proteins of 42 and 28 kDa (fig. 1a). Immunoblotting with polyclonal antibodies against PE, confirmed these data (fig. 1.b).

4. Effect of the GnRH-PE66 chimeric proteins on various cell lines

In the experiments described below, the insoluble fraction of E.coli expressing cells was used as the source of the GnRH-PE66 chimeric protein.
The cytotoxic activity of GnRH-PE66 was tested in various established cell lines: SW-48 colon carcinoma, HepG2 hepatocarcinoma, Caco2 colon carcinoma, OVCAR3 ovarian carcinoma, Hela cervix adenocarcinoma, MDA breast

carcinoma, HT- 29 colon carcinoma. Unless specified, all cell lines were maintained in RPMI 1640 medium, cultured in 100mm petri dishes in a humidified atmosphere of 5%CO2 / 95% air at 37°C. HepG2 and Caco2 were maintained in Eagle's Minimal Essential Medium, Hela cells were maintained in Dulbecco's Modified Eagle's Medium. All media were supplemented with 10% fetal calf serum, 2mM L-glutamin, 100 units/ml of penicillin and 100µg/ml streptomycin. On day 0, cells (10^4 in 0.2 ml culture medium) were seeded in 96 well tissue culture microplates and 24 hours later various concentrations of the GnRH-PE66 were added. After 24 hours incubation [^{3}H]leucine [5µCi per well] was added for an additional 24 hours. At day 3, the plates were stored at -70°C for several hours, followed by a quick thawing at 37°C. Cells were harvested on filters and the incorporated radioactivity was measured with a beta counter. The chimeric protein was found to kill cells in a dose-dependent manner, with considerable variation between cell lines (table 1) ranging from the strong response of HepG2 hepatocarcinoma, SW-48 and Caca2 colon carcinomas (figure 2a) to the intermediate one of ovarian carcinoma OVCAR3, breast carcinoma MDA MB-231, colon carcinoma HT-29 and cervix adenocarcinoma Hela (figure 2b). Although cytotoxicity was measured by inhibition of amino acid incorporation, cell death was reflected in cell number and\or cell necrosis 24 hours following the addition of the chimeric protein.

To confirm the specificity of GnRH-PE66 activity, two other PE based recombinant proteins, expressed and extracted under the same conditions, were used as controls. No substantial growth inhibition was exerted by either PE664Glu, encoded by the mutated full length PE gene, or by PIS2, an unrelated 80 bp sequence fused to PE664Glu. When 15 µg/ml of PE664Glu or PIS2 were added, protein synthesis ranged from a slight increase to 20% inhibition in the different cultures. Growth inhibition resulting from treatment with one of the two proteins was considered nonspecific.

The results are expressed as the percent of the control experiments in which cells were not exposed to any protein (results are summarized in table 1 and in fig. 5).

Table 1: Cytotoxic activity of GnRH-PE66 on various cell lines.

Cell line	Origin	ID_{50} (μg total protein/well)*
Caco2	Colon carcinoma	0.4
HT-29	Colon carcinoma	1.2
SW-48	Colon carcinoma	0.3
OVCAR3	Ovarian carcinoma	3
MDA MB-231	Breast carcinoma	2.3
Hela	Cervix adenocarcinoma	1.8
HepG2	Hepatocarcinoma	0.3

* The ID_{50} values show the effect of the insoluble fraction enriched with the chimeric protein.

5. The effect of GnRH-PE66 on various primary cultures

In order to evaluate the cytotoxic effectiveness of the chimeric proteins on cells resembling the original in vivo tumors as closely as possible and to exclude the possibility that the GnRH-PE66 cytoxicity was a characteristic developed by cells upon prolonged passages, primary cultures were established.

Fresh tissue specimens were obtained from various cancer patients undergoing therapeutic debulking procedures. Control specimens were obtained from donors or patients undergoing diagnostic or therapeutic procedures for non - malignant diseases. All tissue specimens were washed several

times with Leibovitz (L15) medium, and extensively cut with a scalpel. The preparations were then enzymatically proteolysed for 2 hours at 37°C with gentle shaking in Leibovitz medium containing collagenase type I (200u/ml), hyaluronidase (100u/ml), penicillin (1000units/ml), streptomycin (1mg/ml), amphotericin B (2.5 µg/ml) and gentamycin (80 µg/ml). Tissue preparations were centrifuged 10 minutes at 200 x g and the pellets were suspended in RPMI 1640 medium, supplemented with 10% fetal calf serum, penicillin (100u/ml) and streptomycin (100µg/ml) and plated in 100mm petri dishes. Cells were grown for one to three weeks to a density of 8 x 10^6 cells and histopathological diagnoses and cytotoxic assays were performed. Normal leukocytes from peripheral blood and bone marrow aspirates for cytotoxic assays were obtained by diluting whole blood in one volume of phosphate - buffered saline. The diluted sample was placed over an equal volume of Ficoll - Paque and centrefuged for 10 minutes at 200 x g. The cells were resuspended and plated in RPMI 1640 medium containing 20% fetal calf serum, 4 mM l - glutamine, 50 µM ß mercaptoethanol, non essential amino acids, 1mM sodium pyruvate, penicillin (100 units/ml) and streptomycin (100µg/ml).

The cytotoxic effect of the chimeric protein was variable (table 2) with up to three-fold differences in ID50 observed in colon, breast and ovarian primary cultures originated from different patients (figures 3a,c and d respectively).

Table 2: Cytotoxic effect of GnRH-PE66 on various primary cultures

Origin	ID_{50} (µg total protein/well)[a]
Colon carcinoma	0.8 - 2.5[b]
Renal cell carcinoma	1.2
Breast carcinoma	1 - 3[c]
Ovarian carcinoma	1.6 - 3[d]
Bladder carcinoma	no effect
Control cells:	
Colon	no effect[e]
Fibroblasts	" "
Bone marrow	" "
Leukocytes	" "

[a] The ID_{50} values show the effect of the insoluble fraction enriched with the chimeric protein

[b] n=3 [c] n=4 [d] n=2

[e] increasing concentration of GnRH-PE66 did not affect cell growth

In cases where metastasis biopsies could also be obtained, cultures of primary tumors alongside with the metastasis were examined for GnRH-PE66 cytotoxicity. The metastatic cells responded in the same manner, and their ID_{50} were even lower than those of the primary tumors. This may be explained by the high homogeneity of the metastasis culture compared with that of the primary culture.

GnRH-PE66 was also tested on cultures of benign colon peripheral blood bone marrow and skin fibroblasts from healthy donors. The addition of up to 15µg/ml of the

chimeric protein did not result in any measurable dose dependant killing (fig 3f).

6. Histopathological diagnosis of primary cultures

One of the basic questions regarding the veracity of the primary cultures assays is of the epithelial origin of the cells. The tendency of cells in primary culture to lose their epithelial morphology has been described in several carcinomas. To confirm the absence of any substantial amount of "contaminating" fibroblasts, differential staining was performed.
Cells were stained as follows: 10,000 cells were plated on a microscope slide using a cytospin, followed by several minutes incubation at room temperature. Dried slides were fixed by soaking in -20°C cold methanol for 15 minutes and in -20°C cold acetone for a few seconds. Slides were kept at -20°C until staining. Staining was carried out with anti-desmin and anti-keratin antibodies to distinguish fibroblast from epithelial cells, respectively. This staining indicated that the vast majority of the cells a980-100%) were indeed epithelial, even in cases where the cultures exhibited a fibroblast-like shape (fig 4).
Further confirmation was achieved by staining with specific anti tumor marker antigens according to the type of cancer. Formalin fixed sections from the original tumors and the primary cultures cells displayed the same pattern and intensity of staining.

7. Specific binding by GnRH-PE66

To support the findings that colon carcinoma cell lines and primary cultures can be targeted and killed by GnRH-PE66, the ability of plasma membrane fractions from a colon carcinoma cell line to specifically bind GnRH, was examined. The addition of increasing concentrations of GnRH-PE66 chimeric toxin resulted in dose - related displacement of the ^{125}I -

GnRH bound to these membranes. A semiconfluent 100mm dish of the SW-48 colon carcinoma cell line was washed and the cells w re scraped off the plate with a rubber policeman. The collected cells were homogenized in ice - cold assay buffer (10mM Tris - HCl, pH 7.6, 1mM dithiothreitol, 0.15% bovine serum albumin, 1mM EDTA) and centrifuged at 250 x g for 15 minutes (4°C). The resulting pellet was discarded and the supernatant was centrifuged at 20,000 x g for 30 minutes (4°C). The plasma membrane pellet was resuspended in cold assay buffer. Aliquots containing 70 µg plasma membrane protein in a final volume of 100µl, were incubated for 2 hours on ice with 6 x 10^{-6}M (240,000 cpm) ^{125}I-GnRH either in the presence or absence of (10^{-4} - 10^{-10}M) unlabeled GnRH authentic peptide and analog (des - Gly,[d-Ala]-LHRH) or (2.5 x 10^{-5} - 10^{-9}M) GnRH-PE66 chimeric toxin. Following incubation, samples were washed through Whatman GF/C filters with 10 ml of cold assay buffer and counted in a gamma counter.

The addition of increasing concentrations of GnRH-PE66 chimeric toxin resulted in dose related displacement of the ^{125}I-GnRH bound to these membranes. Unlabeled authentic GnRH peptide and the analogue des-Gly10 [D-Ala6]-LHRH produced similar results. As can be seen in figure 5, binding of the labeled GnRH to SW-48 colon carcinoma cell line was specific and displacement by the GnRH-PE66 chimeric toxin was as efficient as that by the GnRH analogue peptide. There was 37% non specific binding.

8. GnRH-PE40 and GnRH-PE66 purification

The pellet of the insoluble fraction was suspended and stirred on ice in denaturation buffer (6M guanidium HCl, 0.1 M Tris HCl, pH 8.6 1mM EDTA 0.05M NaCl and 10mM DDT). After an additional centrifugation, the reduced and denatured protein was diluted 1:100 in refolding buffer (50mM Tris HCl, pH 8, 1mM EDTA, 0.25M NaCl, 0.25 M L-arginine and 5mM DTT) and k pt at 4°C for 48 hours. Refolded protein

solutions were diluted to 8 mS in TE20 buffer (20mM Tris pH 8.0, 1mM EDTA). DEAE Sepharose was added and stirred for half an hour at 4°C before being packed onto a column. Washing of the column was done with 80mM NaCl, in TE20 buffer for GnRH-PE66 and 50mM NaCl in TE20 buffer for GnRH-PE40. Elution was preformed with the linear gradient of 2 x 200ml of 0.08 - 0.35M NaCl, in TE20 (20mM Tris pH 8.0, 1mM EDTA) buffer. The peak fractions were pooled, 0.5M L-arginine was added and stirred cell was used for concentration. (fig. 2 and fig. 3). 3ml of the pooled fractions from the ion exchange column were loaded onto a Sephacryl S-200 HR gel filtration column, in 0.5M NaCl, 0.15M K -phosphate buffer, pH 6.0. The peak fractions were pooled, dialyzed against phosphate saline buffer and kept in aliquotes at -20°C. Purification of GnRH-PE66 and GnRH-PE40 is demonstrated in figures 6 and 7 respectively.

9. Effect of highly purified GnRH-PE chimeric proteins on SW-48 colon carcinoma cell line

The cytotoxic activity of the purified GnRH-PE66 and GnRH-PE40 on SW-48 colon carcinoma cell line was assessed by measuring the inhibition of protein synthesis. The chimeric proteins were found to kill cells in a dose dependent manner. The ID_{50} of the purified GnRH-PE66 chimeric toxin was two to three times lower than the refolded insoluble fraction. The ID_{50} of the GnRH-PE40 purified protein was three to four times lower than the purified GnRH-PE66 (fig. 8).

Claims

1. Targeted chimeric toxins comprising of cell targeting moieties and cell killing moieties for recognizing and for destroying specific cells bearing gonadotropin releasing hormone binding sites, wherein the cell targeting moieties consist of gonadotropin releasing hormone and the cell killing moieties consist of a cell killing Toxin.

2. Targeted chimeric toxins according to claim 1 wherein the specific cells bearing gonadotropin releasing hormone binding sites are selected from malignant carcinoma cells, benign uterine lyomyoma cells, endometrial island cells and pituitary tumor adenoma cells.

3. Targeted chimeric toxins according to claim 1 wherein the cell targeting moiety is an oligonucleotide encoding ten amino acids of a gonadotropin releasing hormone analog and the killing moiety consists of a mutated form of the full length Pseudomonas Exotoxin, providing the protein GnRH-PE66.

4. Targeted chimeric toxins according to claim 1 wherein the cell targeting moiety is an oligonucleotide encoding ten amino acids of a gonadotropin releasing hormone analog and the killing moiety consists of domains II and III of the Pseudomonas Exotoxin, providing the protein GnRH-PE40.

5. A method for the production of targeted chimeric toxin GnRH-PE66 as defined in claim 1 and 3 comprising ligating an oligonucleotide encoding ten amino acids of a gonadotropin releasing hormone analog upstream to a mutated form of PE.

6. A method for the production of cancer cell targeted chimeric toxin GnRH-PE40 as defined in claim 1 and 4 comprising ligating an oligonucleotide encoding ten amino acids of a gonadotropin releasing hormone analog upstream to domains II and III of the PE.

7. Pharmaceutical composition useful for treatment in cancer therapy comprising as active ingredients chimeric toxins as defined in claims 1-4.

8. Chimeric toxins as defined in claims 1-4 and pharmaceutical compositions as defined in claim 7 containing the same as defined in claim 7 for use in the treatment of malignant carcinomas and benign tumors.

9. A method for cancer therapy in mammals by administering to the patient`s body chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7.

10. A method for cancer therapy according to claim 9 wherein the chimeric toxins are administered by systemic administration or by trans cervical washing of the endometrial cavity.

11. A method for treating endometriosis by administering chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7 to the patient`s body.

12. A method for treating endometriosis according to claim 11 wherein the chimeric toxins are administered by peritoneal washings or by ultrasonic guided or laparoscopic intra-endometrial injections or by systemic administeration.

13. A method for treating uterine myomas by administering chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7 to the patient`s body.

14. A method for treating uterine myomas according to claim 13 wherein the chimeric toxins are administered by trans cervical washing of the endometrial cavity.

15. A method for treating pituitary adenomas by administering chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7 to the patient`s body.

16. A method for treating pituitary adenomas according to claim 15 wherein the chimeric toxins are administered intra- thecally.

17. A method for treating BPH by administering chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7 to the patient's body.

18. A method for treating BPH according to claim 17 wherein the chimeric toxins are administeres systemically or by trans - uteral administration of GnRH-PE.

19. A method for treating polycystic breast disease by administering chimeric toxins or their pharmaceutical compositions as defined in claims 1 - 4 and 7 to the patient's body.

20. A method for treating polycystic breast disease according to claim 19 wherein the chimeric toxins are systemically administered.

Figure 1A

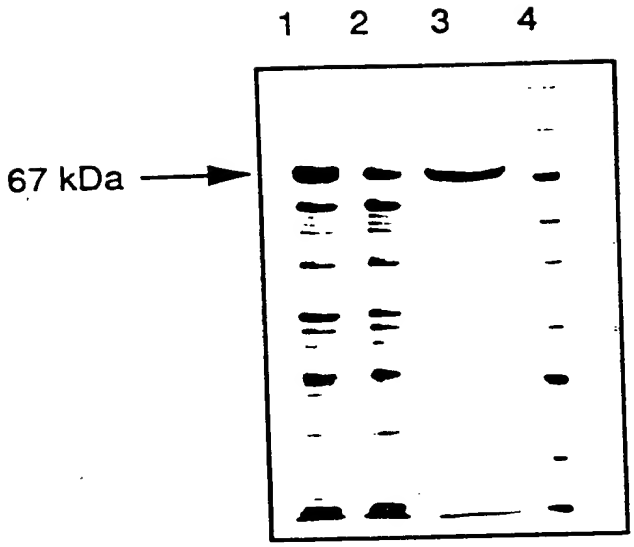


Figure 1B

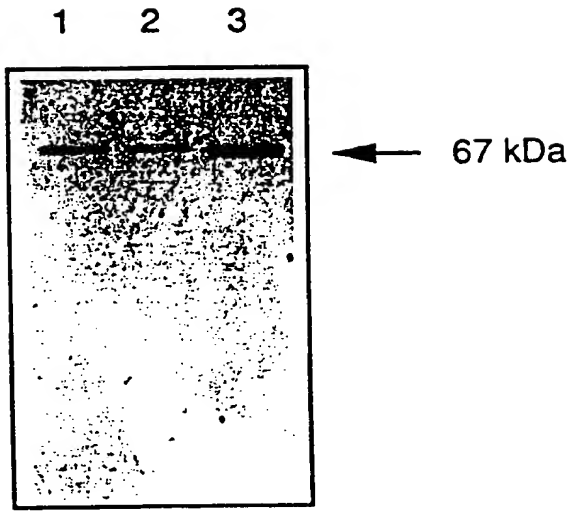


Figure 1C

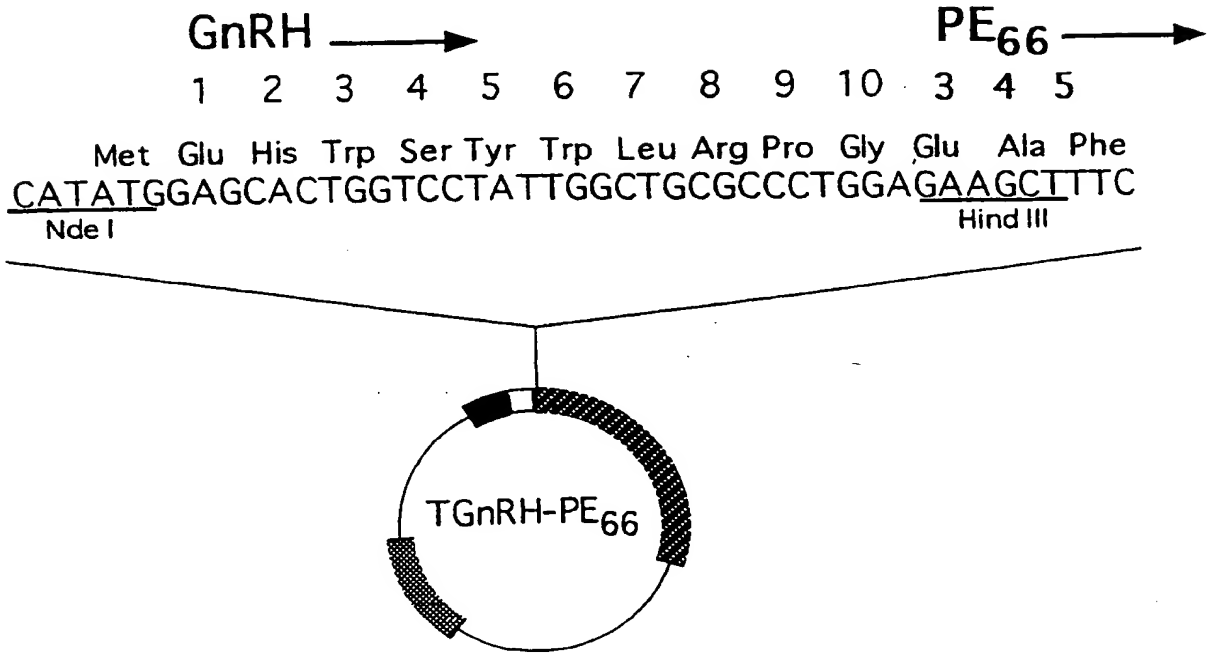


Figure 2A

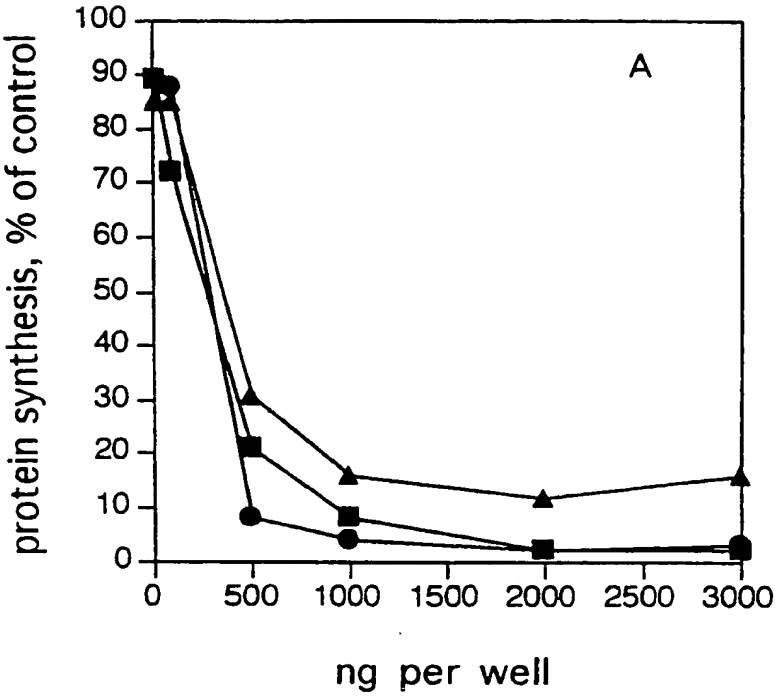


Figure 2B

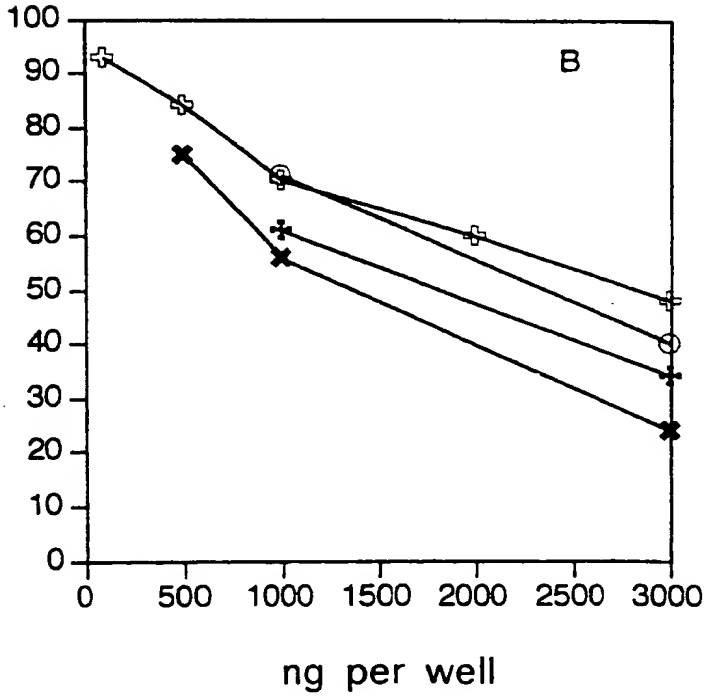


Figure 3C

Figure 3F

Figure 3B

Figure 3E

Figure 3A

Figure 3D

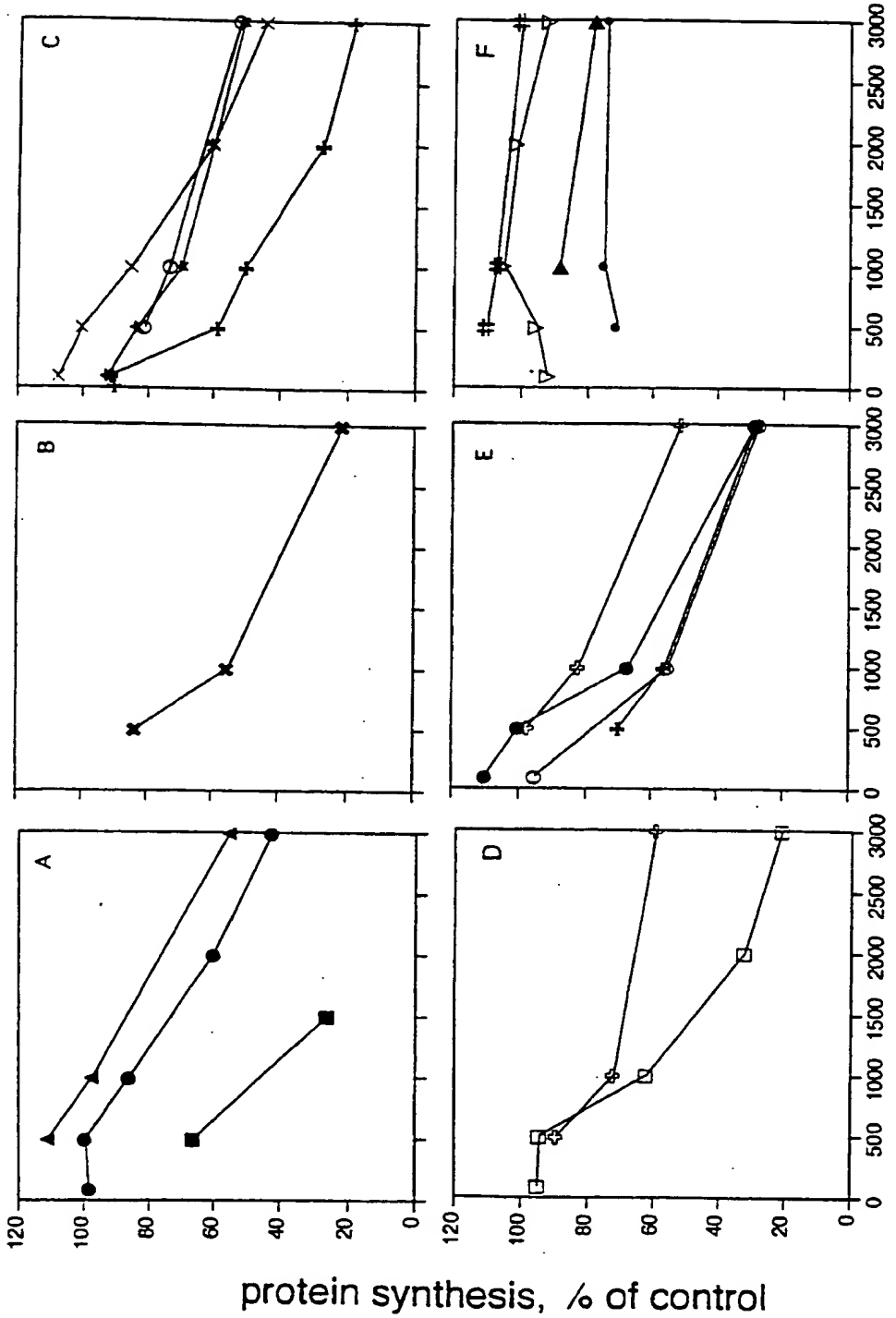


Figure 4A

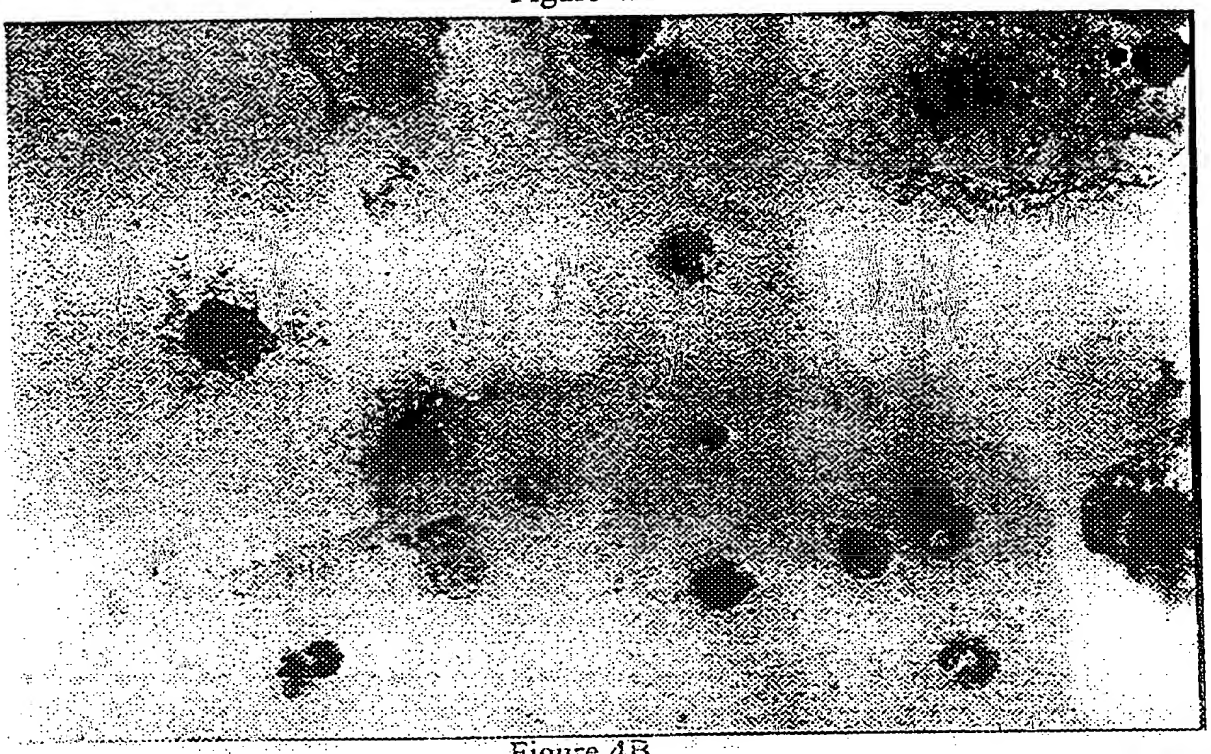
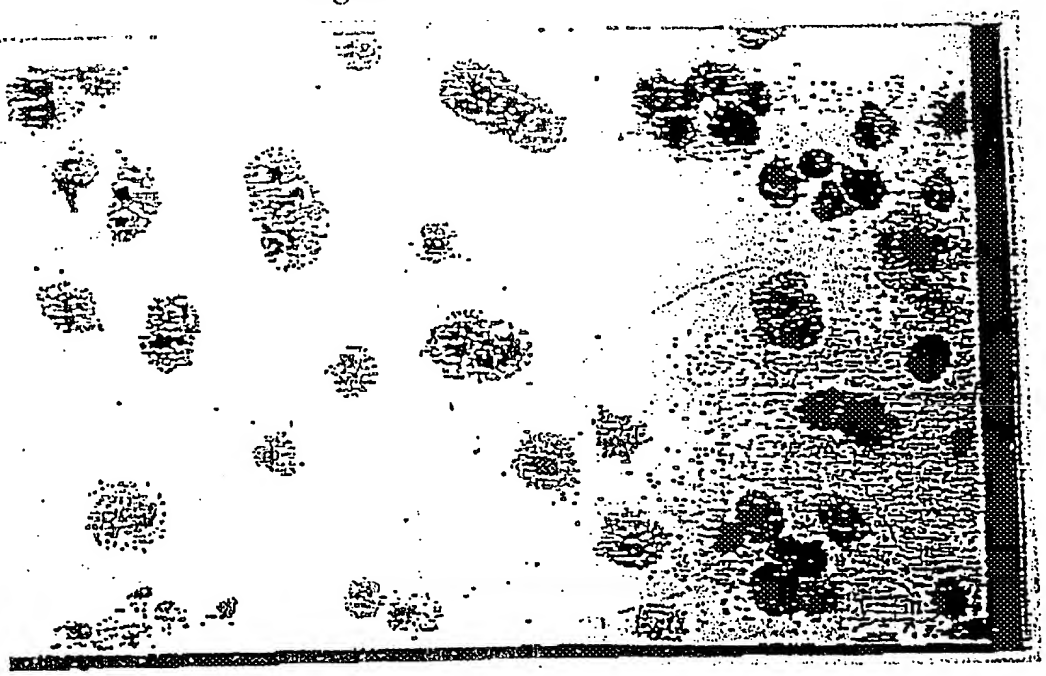


Figure 4B



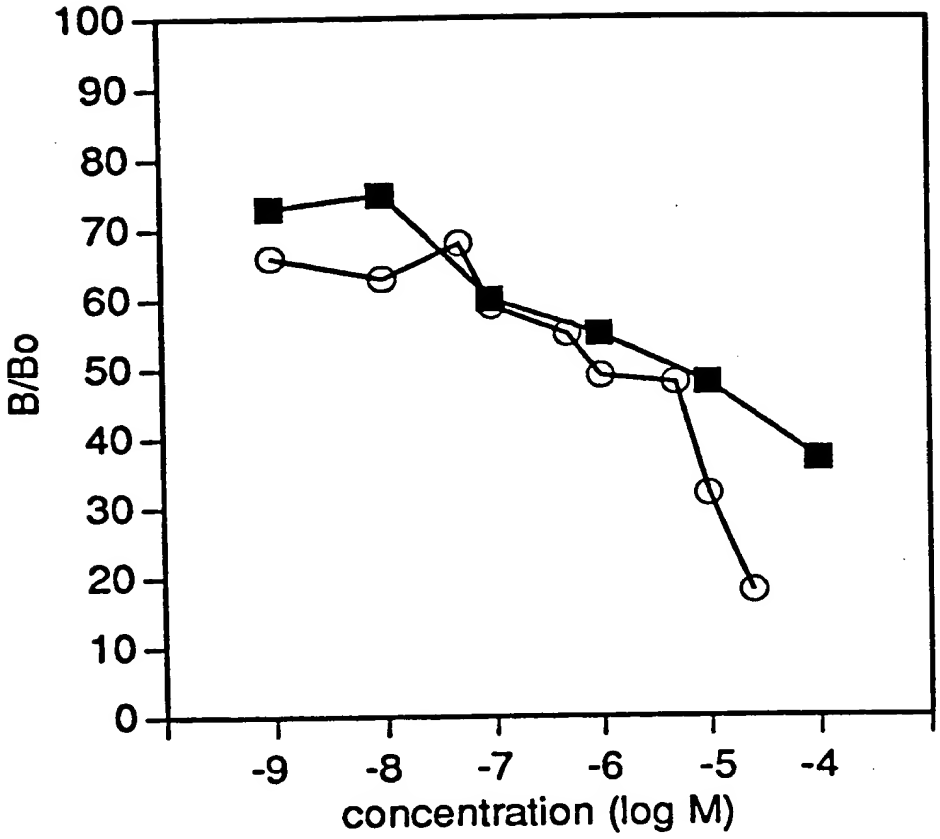


Figure 5

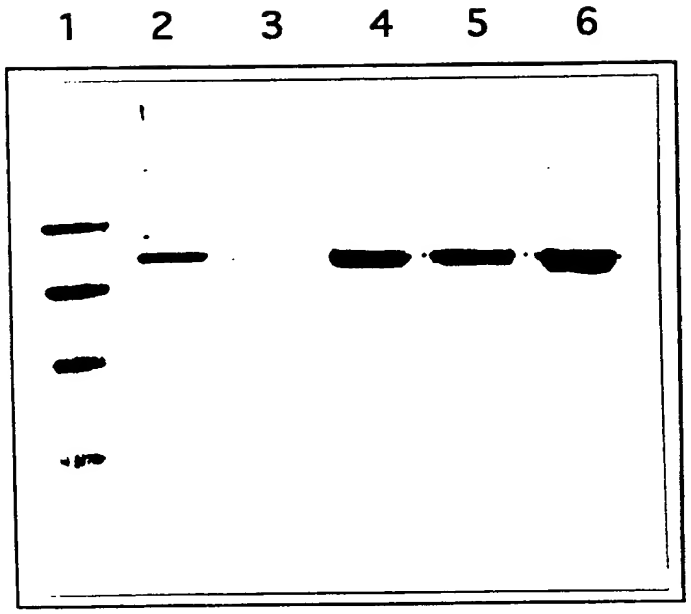


Figure 6

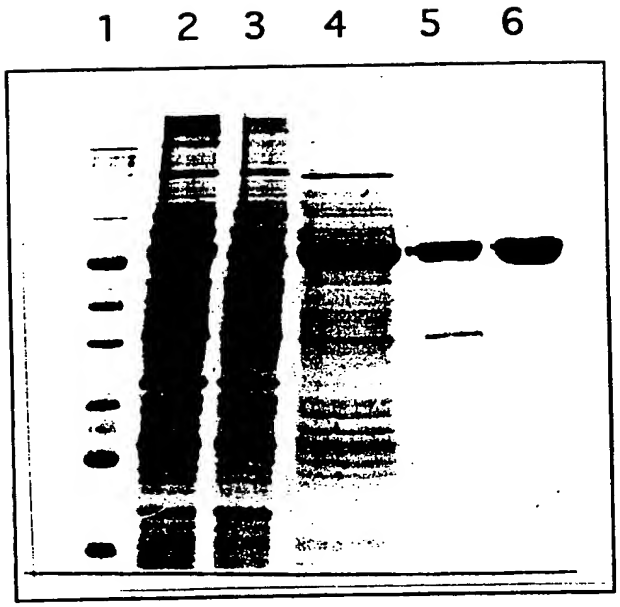


Figure 7

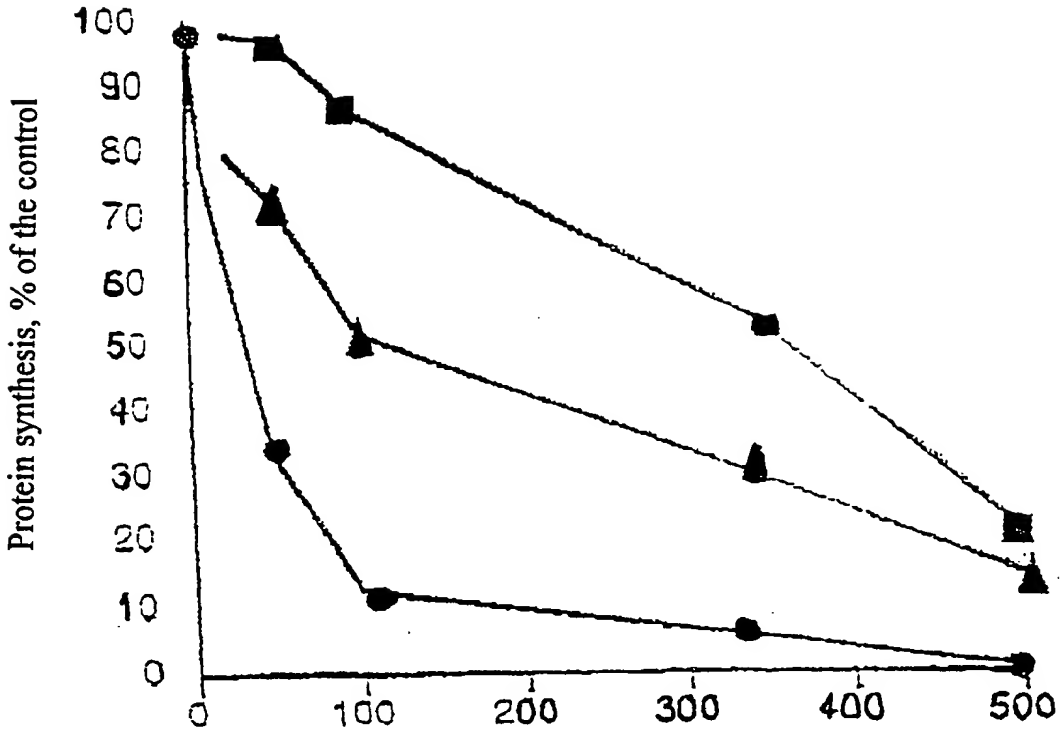

Protein synthesis, % of the control
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500

Figure 8

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(57) Abstract

The present invention relates particularly to neoplastic cells targeted chimeric toxins comprising of cell targeting moieties and cell killing moieties for recognizing and for destroying the neoplastic cells, wherein the cell targeting moieties consist of gonadotropin releasing hormone homologues and the cell killing moieties consist of Pseudomonas Exotoxin A. The present invention further relates to pharmaceutical compositions containing as an active ingredient these neoplastic cells targeted chimeric toxins and to a method for the production of these chimeric toxins. The said invention also relates to a method for cancer therapy, treating malignant carcinoma cells and benign hyperplasia including uterine lyomyoma cells, extra uterian endometrial island cells, benign hyperplasia of prostate and breast and pituitary tumor adenoma cells, by the use of the above-mentioned chimeric toxins.

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INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/IL 97/00180

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NECHUSHTAN, AMOTZ ET AL: "Adenocarcinoma cells are targeted by the new GnRH -PE66 chimeric toxin through specific gonadotropin-releasing hormone binding sites" J. BIOL. CHEM. (1997), 272(17), 11597-11603 CODEN: JBCHA3;ISSN: 0021-9258, 1997, XP002051492 see abstract; figures ---	1-20
X	WO 93 15751 A (MERCK & CO INC) 19 August 1993 cited in the application see claims ---	1-20
P,A	WO 96 24675 A (UNIV SASKATCHEWAN) 15 August 1996 see claims 1-5 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
8 January 1998	-4. 02. 98

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IL 97/00180

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 22364 A (YISSUM RES DEV CO ;FISHMAN ALA (IL); YARKONI SHAI (IL); LORBERBOUM) 26 June 1997 see claims 1,2,7 ---	1-20
X	WO 90 09799 A (UNIV COLORADO RES) 7 September 1990 see page 1, line 3 - line 9 see page 9, line 2 - line 27 see page 14, line 15 - line 34 ---	1-20
X	RUSIECKI, V. K. ET AL: "GnRH -toxin chimera as chemosterilants: Synthesis and conjugation of GnRH analogs to truncated bacterial toxins" PEPT. 1994, PROC. EUR. PEPT. SYMP., 23RD (1995), MEETING DATE 1994, 765-766. EDITOR(S): MAIA, HERNANI L. S. PUBLISHER: ESCOM, LEIDEN, NETH. CODEN: 63MBAO, 1995, XP002051493 see the whole document -----	1

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 97/00180

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 9-20
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCT/IL 97/00180

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9315751 A	19-08-93	AU 3665293 A	03-09-93
		ZA 9300988 A	20-09-93
WO 9624675 A	15-08-96	AU 4477796 A	27-08-96
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		EP 0808369 A	26-11-97
WO 9722364 A	26-06-97	AU 1070797 A	14-07-97
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